#12

EXHIBIT B

Development of Th1 and Th2 Populations and the Nature of Immune Responses to Hepatitis B Virus DNA Vaccines Can Be Modulated by Codelivery of Various Cytokine Genes[1]

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In this study, we provide direct evidence that the magnitude and nature of the immune response to a DNA vaccine can be differentially regulated by codelivery of various mouse cytokine genes. Mice immunized with a hepatitis B virus (HBV) DNA vaccine and the IL-12 or IFN-γ gene exhibited a significant enhancement of Th1 cells and increased production of anti-HBV surface IgG2a Ab, as well as a marked inhibition of Th2 cells and decreased production of IgG1 Ab. In contrast, coinjection of the IL-4 gene significantly enhanced the development of specific Th2 cells and increased production of IgG1 Ab, whereas Th1 differentiation and IgG2a production were suppressed. Coinjection of the IL-2 or the granulocyte-macrophage-CSF gene enhanced the development of Th1 cells, while the development of Th2 cells was not affected, and the production of IgG1 and IgG2a Ab were both increased. The CTL activity induced by HBV DNA vaccination was most significantly enhanced by codelivery of the IL-12 or IFN-γ gene, followed by the IL-2 or granulocyte-macrophage-CSF gene, whereas codelivery of the IL-4 gene suppressed the activity. When challenged with HBV surface Ag (HBsAg)-expressing syngeneic tumors, significant reduction of tumor growth was observed in mice that were coadministered the IL-12 gene but not the IL-4 gene. Taken together, these results demonstrate that application of a cytokine gene in a DNA vaccine formulation can influence the differentiation of Th cells as well as the nature of an immune response and may thus provide a strategy to improve its prophylactic and therapeutic efficacy. *The Journal of Immunology*, 1998, 160: 1320–1329.

DNA vaccines contain gene(s) for an antigenic portion of a virus, such as the core protein or the envelope protein, usually under the transcriptional control of a viral promoter (1–3). Direct injection of the plasmid DNA in vivo results in prolonged expression of viral proteins in the host and may thus mimic the action of attenuated vaccines. An important advantage of this novel vaccination method is that the in vivo-synthesized viral protein can enter both the MHC class I and class II Ag-processing pathways to activate specific immunization. Many animal models of infectious diseases have been reported (4–15) in which DNA vaccines induce a broad range of immune responses, including Abs, $CD8^+$ CTL, $CD4^+$ Th cells, and protective immunity against challenge with the pathogen. Application of this genetic vaccination approach has also been extended to the treatment of cancers (16–21) as well as allergic (22–24) and autoimmune diseases (25).

The protective value of the immune response is highly dependent upon the types of cytokines produced by $CD4^+$ Th cells. This concept was first clearly demonstrated in the in vivo study of the immune response to *Leishmania major* infection in a murine model, in which resistance or susceptibility to the parasite was dependent on the development of respective Th1 or Th2 effector cells (26, 27). Th1 cells that produce IL-2 and IFN-γ induce activation of macrophages, delayed-type hypersensitivity, and production of IgG2a Ab (28, 29). In contrast, Th2 cells that predominantly produce IL-4, IL-5, IL-10, and IL-13 promote development of eosinophilia as well as generation of Abs of IgG1 and IgE isotype (28, 29). Indeed, Th1 and Th2 cells constitute a dynamic and mutually inhibitory network that tends to support distinct elements of the immune system. The best defined among the stimuli that direct the development of naive CD4 cells into Th1 or Th2 effectors are the cytokines to which the naive cells are exposed during antigenic stimulation. Thus, IL-4 plays a critical role in the priming of Th2 cells (30), whereas IL-12 alone (31) or together with IFN-γ (32) stimulates differentiation of naive T cells into the Th1 lymphokine-producing phenotype.

We have shown previously that coexpression of IL-2 and hepatitis B virus (HBV)[3] envelope protein within the same plasmid vector resulted in a dramatic increase in its ability to induce humoral and cellular immune responses to HBsAg (33). The IL-2 adjuvant activity also helps the HBV DNA vaccine elicit high anti-HBs titers in animals that usually fail to respond to rHBsAg vaccination, a phenomenon that is closely related to certain MHC haplotype (34, 35). In addition, splenocytes derived from mice that received plasmids coexpressing IL-2 and the HBV envelope protein produced much stronger Th1-like responses than those from

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[3] Abbreviations used in this paper: HBV, hepatitis B virus; HBsAg, hepatitis B virus surface Ag; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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mice that had been inoculated with plasmids encoding the envelope protein alone. In the present report, we investigate whether coadministration of other cytokine genes is able to modulate the immune response by favoring the development of Th1 vs Th2 cells. We found that mice given injections of the plasmid encoding HBsAg together with plasmids encoding IL-12 or IFN-γ promoted Th1 and suppressed Th2 differentiation. Conversely, coinjection of a plasmid expressing IL-4 promoted Th2 and suppressed Th1 differentiation. Coadministration of the IL-2 or GM-CSF gene mainly enhanced Th1 cell differentiation, and left Th2 cell development unaffected. In accordance with the different Th populations induced by DNA vaccination under influence of various cytokine genes, the Ab isotype, CTL activity, as well as the in vivo antitumor effect, were also substantially altered.

Materials and Methods

Construction of expression vectors

The plasmid vector pS encoding HBV major envelope proteins was constructed previously (33). This eukaryotic expression vector was modified from plasmid pcDNA3 (Invitrogen, San Diego, CA) containing the CMV early promoter/enhancer sequence and the polyadenylation and 3′ splicing signals from bovine growth hormone. The cDNA of murine IFN-γ, IL-4, and GM-CSF were obtained by reverse transcription and PCR amplification of RNA derived from about 1×10^6 mouse (C3H/HeN) splenocytes stimulated for 4 h, 8 h, and 24 h, respectively, with 5 ng/ml of PMA (Sigma Chemical Co., St. Louis, MO) and 5 μg/ml of Con A (Sigma) in culture medium. The fragment containing the murine IL-2 coding sequence was generated by PCR from plasmid pmut-1 (ATCC 37553; American Type Culture Collection (ATCC), Rockville, MD). The upstream PCR primer for each cytokine gene contains a *Bam*HI site, and the downstream primer contains an *Eco*RI site. PCR products of IL-2, IL-4, GM-CSF, and IFN-γ were digested with *Bam*HI and *Eco*RI, gel purified, and inserted between the *Bam*HI and *Eco*R1 sites of pcDNA3 to generate pIL-2, pIL-4, pGM, and pIFN-γ, respectively. The fragments containing the p35 and p40 coding sequences of murine IL-12 were generated by PCR from plasmids BLpSV35 and BLpSV40 (kindly provided by Michale T. Lotze, University of Pittsburgh, Pittsburgh, PA), respectively, and cloned into a bicistronic *plasmid*, pTCAE (33), under the control of discrete CMV promoters. Plasmid DNA was purified from transformed *Erichia coli* strain DH5α by Qiagen Plasmid Giga Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at −70°C as pellets. The DNA was reconstituted in sterile saline at a concentration of 1 mg/ml for experimental use.

Cell transfection and cytokine gene expression

The expression of cytokine genes by the plasmid DNA was performed in a transient transfection assay using Lipofectamine (Life Technologies, Gaithersburg, MD) as specified by the manufacturer. Briefly, C2C12 mouse myoblasts (1×10^5 cells; ATCC 1772) were cultured in DMEM plus 10% FCS (DMEM-10) in a six-well tissue culture plate until the cells reached approximately 50 to 80% confluence. Three micrograms of plasmid DNA was mixed with 20 μl of Lipofectamine in 200 μl of OPTI-MEM medium (Life Technologies) at room temperature. Following a 20-min incubation, the DNA-liposome complexes were diluted in 800 μl of OPTI-MEM and slowly added to cells, which had been prewashed twice with 5 ml of OPTI-MEM. After a 16-h incubation, the DNA-liposome complexes were removed, 2 ml of DMEM-10 was added to each well, and incubation was continued for another 48 h. The supernatant of each well was collected and stored at −80°C for cytokine analysis.

Cytokine ELISAs and proliferation assays

The cytokine activity present in the supernatant of plasmid DNA-transfected cells was assayed for its ability to support the proliferation of appropriate responsive cells: IL-2 and IL-4 were tested on HT-2 cells (36), GM-CSF was measured on NFS-60 cells (37), and IL-12 was determined on activated lymphoblasts. The HT-2 and NFS-60 cells were grown in RPMI 1640 containing 10% FCS (RPMI 10) supplemented with 1% supernatant of transfectoma cell lines Id-IL-2 (38) and Id-MoGM (39), which produce Id-cytokine fusion proteins containing murine IL-2 and GM-CSF activity, respectively. To perform the proliferation assay, samples were added in triplicate to 96-well plates in RPMI 10 with 5,000 HT-2 or NFS-60 cells to a total volume of 0.1 ml and incubated in a humidified incubator for 16 to 24 h at 37°C and 5% CO_2. IL-12 proliferation assay was performed as previously described (40). Briefly, the Con A-activated lymphoblasts were prepared by culturing C57BL/6 splenocytes in TCM medium (Celox Laboratory, Hopkins, MN) containing 2% FCS, 20 ng/ml of human rIL-2 (Endogen Inc., Cambridge, MA) and 2 μg/ml of Con A (Sigma) at a density of 2×10^6 cells/ml for 2 to 3 days. The lymphoblast cells were then harvested and cultured at 50,000 cells/well with test samples in TCM plus 5% FCS for 24 h. After incubation, 1 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added to each well in 50 μl of growth medium, and cells were harvested 4 to 6 h later using a FilterMate (Packard, Meriden, CT) automatic cell harvester; the incorporated radioactivity was determined using TopCount microplate scintillation counter (Packard). The concentration of cytokines in the samples was determined from the standard curves generated using recombinant murine IL-2, IL-4, GM-CSF, or IL-12, all of which were purchased from PharMingen (San Diego, CA).

ELISA detection systems (PharMingen) were also used to screen for the presence of IFN-γ, IL-2, and IL-4 cytokines in test samples. The capture Abs for murine IFN-γ, IL-2, and IL-4 were R46A2 (rat IgG1), JES6-1A12 (rat IgG2a), and BVD4-1D11 (rat IgG2b), respectively. The detection biotinylated Abs for IFN-γ, IL-2, and IL-4 were XMG1.2 (rat IgG1), JES6-5H4 (rat IgG2b), and BVD6-24G2 (rat IgG1), respectively. Briefly, microtiter plates were coated with 50 μl of anti-cytokine capture mAb at the concentration of 2 μg/ml overnight at 4°C. The plates were washed twice with PBS/Tween and blocked with 200 μl per well of 10% bovine calf serum in PBS for 2 h at room temperature. Then, the plates were washed twice and incubated with duplicates of serially diluted samples and standards overnight at 4°C. One hundred microliters of the biotinylated anti-cytokine mAb at a concentration of 2 μg/ml was added to each well and incubated at room temperature for 1 h. The plates were then washed six times. 100 μl of avidin-phosphatase (1:500; PharMingen) was added, and the mixture was incubated at room temperature for 30 min. Following multiple final washings, the color was developed with *p*-nitrophenyl phosphate (Sigma) as the substrate, and absorbance at 405 nm was measured using an ELISA plate reader. The concentration of cytokines in the samples was determined from the standard curve.

Immunization of mice

Female BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from the Laboratory Animal Facility, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan. All mice were immunized at 6 to 8 wk of age as described previously (33). In brief, groups of three to five mice were anesthetized and injected i.m. in the left hind thigh muscle with a mixture of 100 μg of pS and 100 μg of pcDNA3, pIL-2, pIL-4, pGM, pIFN-γ, or pIL-12. Mice immunized with 200 μg of pcDNA3 served as negative controls. In some experiments, mice were immunized with 100 μg of pS in the left thigh and 100 μg of each cytokine vectors or the control plasmid in the right thigh. For protein immunization controls, mice were given s.c. injections in the base of the tail with 4 μg of yeast-derived rHBsAg (41) in CFA (Life Technologies, Grand Island, NY).

Ab assay

Serum samples were collected by tail bleeding at different times, beginning 1 wk after immunization, and analyzed for the presence of HBsAg-specific Abs. Microtiter plates were coated with 5 μg/ml of yeast-derived rHBsAg. After incubation with 200 μl of 5% powdered milk in PBS on each well for 1 h to prevent nonspecific binding, 50 μl of serial dilutions of test sera was added to each well and incubated overnight at 4°C. After the samples were washed with PBS, bound proteins were detected with horseradish peroxidase-conjugated goat anti-mouse IgM (1:2000, Cappel-Organon Teknika, Veedijk Belgium) or anti-mouse IgG (1:2000; Cappel). Color was generated by adding 2,2′-azino-bis(ethylbenzthiozoline sulfonic acid), and absorbance at 492 nm was measured on an ELISA reader. For measurement of IgG1 and IgG2a anti-HBs isotypes, biotin-conjugated rat anti-mouse IgG1 (1:500, PharMingen) and rat anti-mouse IgG2a (1:500, PharMingen) were used as detectors. Avidin-alkaline phosphatase (1:1000, PharMingen) was then added. Color was developed with the addition of *p*-nitrophenyl phosphate (Sigma), and absorbance at 405 nm was measured. Concentrations of total IgG anti-HBs Abs in serum samples were estimated from the standard curve generated by a HBsAg-specific mAb (H25B10, ATCC CRL-8017) and expressed as μg/ml for IgG. For measurement of IgM, IgG1, and IgG2a Abs, readings were referenced to a standard serum pooled from four mice given i.p. injections of 2 μg of rHBsAg with CFA and bled 4 wk after immunization. The standard curves were generated using the pooled anti-HBs sera, and results were expressed as arbitrary units per milliliter (U/ml; 1 U ≈ 50% maximum OD).

Flow cytometric analysis

The populations of splenocytes derived from immunized animals were analyzed by flow cytometer. Splenocytes were stained with FITC-conjugated anti-mouse CD4 or anti-mouse CD8α, combined with phycoerythrin-conjugated anti-Thy1.2; all Abs were purchased from PharMingen. Stained cells were analyzed on a FACSCallibur (Becton Dickinson, Mountain View, CA).

Assays for lymphocyte proliferation and cytokine secretion

To determine whether HBsAg-specific lymphoproliferative responses were induced in immunized aimals, spleens were removed 2 wk after immunization to make single-cell suspensions. $CD4^-$-depleted and $CD8^-$-depleted immune splenocytes were prepared by immunomagnetic depletion of $CD4^-$ and $CD8^-$ lymphocytes, respectively, and replaced with the same number of purified $CD4^+$ or $CD8^+$ cells isolated from naive mice. The immunomagnetic depletion or enrichment of $CD4^+$ and $CD8^+$ lymphocytes was performed by a magnetic activated cell sorter (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), using microbeads conjugated with rat anti-mouse CD4 and rat anti-mouse CD8 mAbs (Miltenyi Biotec), respectively, according to the manufacturer's instructions. To perform the lymphoproliferative assay, 100 μl of 2×10^6/ml unfractionated, $CD4^-$-depleted, or $CD8^-$-depleted splenocytes in complete RPMI 10 were added to each well in 96-well flat-bottom plates. Stimulated wells received purified rHBsAg at a concentration of 30, 10, or 1 μg/ml; transferrin (120 μg/ml, Sigma) served as a negative control Ag and Con A (5 μg/ml, Sigma) as a positive mitogenic control. Control wells received cells only. Cells in all the wells were cultured in a total volume of 200 μl of medium. After 4 days in culture, the cells were pulsed with [^{3}H]thymidine (1 μCi/well) for 18 h and harvested with FilterMate (Packard) and the incorporated radioactivity was determined by TopCount (Packard). The stimulation index was calculated as the mean cpm of the stimulated wells divided by the mean cpm of the control wells.

To measure cytokine secretion, splenocytes were cultured as described above with the same panel of Ags or mitogen over the same range of concentrations with the exception that after 3 days in culture, cell-free supernatants were harvested and assayed immediately or stored at −80°C. These supernatant were screened for the presence of IFN-γ, IL-2, and IL-4 using ELISA detection systems as described in the previous section.

Cytotoxicity assays

BALB/c mice were immunized i.m. with 100 μg of pS and 100 μg of each of the cytokine vectors or the control plasmid. Spleens were removed 3 wk after immunization to measure the CTL activity. $CD4^-$-depleted and $CD8^-$-depleted immune splenocytes were prepared as described in the previous section. Single-cell suspensions of unfractionated, $CD4^-$-depleted or $CD8^-$-depleted splenocytes were treated with 5 ml per spleen of ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) for 5 min at room temperature to remove RBC. HBsAg-expressing P815 cells, a murine mastocytoma line ($H\text{-}2^d$, DBA/2 mice), and CT26 cells, a murine colon carcinoma ($H\text{-}2^d$, BALB/c mice), were generated by permanent transfection with 20 μg of plasmid pS, selected for G418-resistant clones, and screened for the expression of HBsAg by ELISA and immunostaining. One of the HBsAg-expressing clone from each transfected cell line was selected and designated as P815/S and CT26/S, respectively. To perform the cytotoxic assay, responder splenocytes (4×10^6 per well) were first stimulated with irradiated (8000 rad) P815/S or CT26/S transfectants (4×10^5 per well) in RPMI 10 and 20 U/ml human rIL-2 (Genzyme Corp., Cambridge, MA) in 24-well plates for 4 days at 37°C. A chromium release assay was employed to measure the ability of in vitro-stimulated responder cells to lyse P815/S and CT26/S as well as the non-transfected parental cells. Target cells (1×10^6 in 0.1 ml RPMI 10) were labeled with 0.1 mCi radiolabeled sodium chromate (Amersham) in 0.1 ml normal saline for 2 h at 37°C, washed three times with RPMI 10, and resuspended at a concentration of 5×10^4 per ml in RPMI 10. A threefold serial dilution of 100 μl of stimulated responder splenocytes (starting from 5×10^5 cells) was added to individual wells containing 100 μl of labeled target cells (5000 cells). In some experiments, the anti-CD4 mAb (GK1.5, ATCC TIB-207) or anti-CD8 mAb (53-6.72, ATCC TIB-105) was added to the culture medium during the cytotoxic assay at a concentration of 10 μg/ml. After a 6-h incubation at 37°C, 100 μl of culture supernatant was collected for gamma radiation counting. The percent specific lysis was calculated as [(experimental release − spontaneous release)]/(total release − spontaneous release)] × 100. Spontaneous release represents the amount of radioactivity released from target cells without the addition of effector cells. Total release represents the amount of radioactivity released following lysis of target cells after the addition of Triton X-100 to 1.0%.

In vivo tumor protection

Three weeks after injection of plasmid pS and various cytokine genes, individual groups of mice were challenged s.c. in the left lateral flank with 1×10^5 CT26 cells or the HBsAg gene-transfected CT26/S cells. Tumor growth was measured every second or third day.

Results

Construction and expression of cytokine vectors

The presence of certain cytokines during antigenic stimulation has been shown to be of decisive importance in directing the development of naive $CD4^+$ cells into Th1 or Th2 effectors and thus may determine the outcome of many infectious and autoimmune diseases. DNA vaccination is a recently developed vaccine technology and has been reported as able to induce humoral and cellular immunities in many different disease models. To study whether the phenotype of immune responses to a DNA vaccine can be altered by cytokines, a previously described plasmid vector pS (33), which encodes HBsAg, was used as a model system. Since local expression of cytokines at the site of immune interaction is important to mediate its function, we hypothesized that codelivery of a cytokine vector with the HBV DNA vaccine might achieve this purpose. The cytokine-producing vectors pIL-2, pIL-4, pGM, pIFN-γ, and pIL-12 encoding murine IL-2, IL-4, GM-CSF, IFN-γ, and IL-12, respectively, were constructed as described in *Materials and Methods*. To test whether the various cytokine vectors can produce biologically active cytokines, mouse C2C12 myoblasts were transiently transfected with each of the vectors. Two days after transfection, the amount of cytokines present in the culture supernatant was analyzed by ELISA (for IFN-γ) or proliferation assays using appropriate responsive cells (HT-2 cells for IL-2 and IL-4; NFS-60 cells for GM-CSF and mitogen-activated lymphoblast for IL-12). Plasmids pIL-2, pIL-4, pGM, pIFN-γ, and pIL-12 produced 45.8 ± 4.8, 77.6 ± 6.8, 48.4 ± 6.5, 11.3 ± 0.3, and 17.6 ± 5.3 ng/ml of cytokine proteins, respectively, each with its particular biologic activity.

Codelivery of cytokine vectors enhances T cell proliferative responses to pS DNA vaccination

The enhancing effect of various cytokines on T cells was then examined. Groups of C57BL/6 mice were given i.m. injections of pS alone, or a mixture of pS plus a cytokine vector or the parental vector pcDNA3. Mice that received pcDNA3 served as negative controls. Mice immunized with rHBsAg were also included as protein immunization controls. At 2 wk after immunization, splenocytes were examined for proliferation in response to specific Ag stimulation. Both plasmid DNA and rHBsAg immunization elicited significant proliferative responses over a range of concentrations (1–30 μg/ml; Table I). Coinjection of plasmids expressing IL-2, GM-CSF, IFN-γ, or IL-12 with pS enhanced the cellular proliferation by two- to threefold, while plasmid encoding IL-4 gave a less significant increase in the proliferative response (Table I). The enhancement in cellular proliferation by cytokine vectors cannot be attributed simply to the intrinsic adjuvant effect of plasmid DNA as previously reported (42), since splenic lymphocytes derived from mice inoculated with pS plus pcDNA3 proliferated at similar levels as those immunized with pS alone. Mice vaccinated with the control pcDNA3 vector did not respond to HBsAg, and all mice failed to respond to transferrin included as a control Ag, indicating that the observed T cell proliferation was HBsAg specific. We also found that the adjuvant effect conveyed by the cytokine vector was only observed when the DNA vaccine was coinjected with the cytokine vector. Mice immunized with pS on the left thigh and cytokine cDNA vectors on the right thigh did not enhance the HBsAg-specific proliferative response (data not

Table I. *Effect of coexpression of various cytokines on HBsAg-specific T cell responses induced by the HBV DNA vaccine*

Antigen[a]	Dose (μg)	T cell Stimulation Index with Stimulant[b] HBsAg 30 μg/ml	HBsAg 10 μg/ml	HBsAg 1 μg/ml	Transferrin (120 μg/ml)
rHBsAg	4	13.6 ± 0.4	10.0 ± 1.3	4.1 ± 0.2	0.6 ± 0
pcDNA3	200	1.2 ± 0	1.4 ± 0	1.1 ± 0	1.2 ± 0
pS	100	7.4 ± 0.4	4.5 ± 0.7	2.2 ± 0.5	1.7 ± 0.4
pS + pcDNA3	100 + 100	8.8 ± 0.4	5.3 ± 0.2	2.4 ± 0.3	1.1 ± 0
pS + pIL-2	100 + 100	18.6 ± 1.1	12.3 ± 0.4	4.2 ± 0.3	1.2 ± 0
pS + pIL-4	100 + 100	12.2 ± 1.4	6.3 ± 0.7	3.1 ± 0.4	0.9 ± 0
pS + pGM	100 + 100	16.7 ± 0.6	9.7 ± 0.5	3.9 ± 0.2	1.0 ± 0
pS + pIFN-γ	100 + 100	20.8 ± 2.9	12.1 ± 2.4	5.2 ± 0.3	0.7 ± 0
pS + pIL-12	100 + 100	25.5 ± 2.5	15.0 ± 2.1	4.2 ± 0.3	1.2 ± 0

[a] C57BL/6 mice were given i.m. injections of 100 μg of pS or a mixture of 100 μg of pS plus 100 μg of either the parental vector (pcDNA3) or one of the different cytokine vectors. Mice receiving 200 μg of pcDNA3 served as negative controls. Mice injected s.c. from the tail base with 4 μg of rHBsAg in CFA served as protein controls. At 2 wk after immunization, splenocytes pooled from three immunized mice were used in proliferation assays.

[b] Values are from one representative experiment of three performed and are presented as the mean stimulation index for triplicate wells ± SD.

shown), indicating that the presence of cytokines at the site of immune interaction was important for this enhancement.

The splenic lymphocyte populations of immunized animals were evaluated by flow cytometric analysis. Codelivery of cytokine genes did not change the proportions of $CD4^+$ and $CD8^+$ Th cells in the plasmid DNA-vaccinated animals. The frequency of $CD4^+$ lymphocytes in mice coinjected with pS and pIL-2, pIL-4, pGM, pIFN-γ, or pIL-12 was 32.7, 31.9, 31.1, 27.9, and 33.6%, respectively, which was comparable with that from mice immunized with pS plus pcDNA3 (30.8%) or pcDNA3 alone (30.9%). The frequency of $CD8^+$ lymphocytes was also similar among groups of mice injected with or without cytokine vectors: 13.3% (pS + pIL-2), 14.2% (pS + pIL-4), 11.7% (pS + pGM), 12.0% (pS + pIFN-γ), and 13.6% (pS + pIL-12) vs 11.0% (pS + pcDNA3) and 12.7% (pcDNA3). To determine which lymphocyte population was responsive to HBsAg, spleen cells showing proliferative responses were further tested by immunomagnetic depletion of $CD4^+$ or $CD8^+$ lymphocytes. In all immunized groups, depletion of $CD4^+$ cells significantly decreased the HBsAg-specific proliferative response, whereas depletion of $CD8^+$ cells had little effect on this response (Fig. 1), indicating that the bulk of the proliferative responses could be accounted for by $CD4^+$ cells.

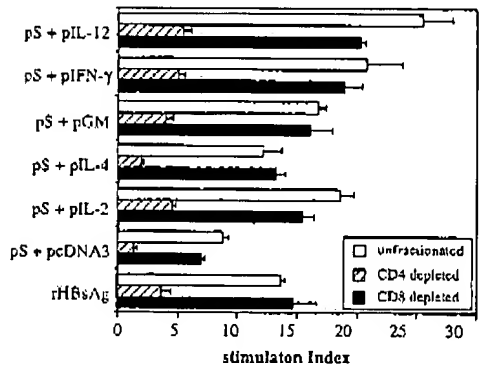

FIGURE 1. HBsAg-specific proliferative responses are mediated by $CD4^+$ Th cells. Animals were treated as described in the legend to Table I. The preparation of $CD4^+$-depleted and $CD8^+$-depleted splenocytes is detailed in *Materials and Methods*. Values are presented as the mean stimulation index for triplicate wells ± SD.

The magnitude of Ab responses to HBV DNA vaccination is altered by codelivery of various cytokine genes

To establish whether the humoral responses to the HBV DNA vaccine can be affected by simultaneous expression of cytokine genes, groups of five mice were given i.m. injections of a mixture of pS and various cytokine vectors. Mice receiving 4 μg of rHBsAg were also included as a comparison. Serum from each mouse was obtained at week 4 and week 8 following DNA or protein injection for analysis of IgM and IgG anti-HBs Ab responses, respectively. As shown in Figure 2, both plasmid pS and rHBsAg elicited significant titers of HBsAg-specific IgM and IgG Abs. Coinjection of IL-2, IL-4, GM-CSF, IL-12, or IFN-γ genes with pS all resulted in much stronger IgM anti-HBs Ab compared with mice receiving pS plus the parental vector (Fig. 2*A*). The IgG anti-HBs Ab were enhanced two- to threefold in mice that had received pS together with pIL-2, pIL-4, pGM, or pIL-12 compared with those immunized with pS plus the parental vector (Fig. 2*B*). In contrast, we found that mice given injections of plasmids pS and pIFN-γ showed decreased IgG anti-HBs titers compared with the control mice. We also performed kinetic studies on the DNA vaccine-induced humoral responses and found that the adjuvant effect conveyed by the cytokine vector was observed only at the early time intervals, from week 4 through 20, after DNA immunization (data now shown). The influence was subsequently decreased, and by week 35, the difference in anti-HBs titers between groups immunized with and without cytokine coexpression became negligible. Mice that were given injections of pS and the cytokine vector on different thighs showed no alterations in the anti-HBs Ab titers (data not shown), indicating the importance of colocalization of cytokines with Ag to exert their adjuvant effects.

Differentiation of $CD4^+$ Th cell into Th1 or Th2 phenotype can be altered by codelivery of various cytokine vectors

It is known that the subsets of Th cells can be distinguished by the pattern of cytokines that they produce. Th1 cells produce IFN-γ and IL-2, and Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (27, 43–45). To study the effect of cytokine coexpression on the development of Th cells induced by DNA vaccination, cytokines profiles released from HBsAg-stimulated splenocytes were compared among groups of mice immunized with pS with or without cytokine gene coinjection. Spleen cells from animals immunized with plasmid pS plus pcDNA3 or rHBsAg produced both Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines (Fig. 3). Coexpression of

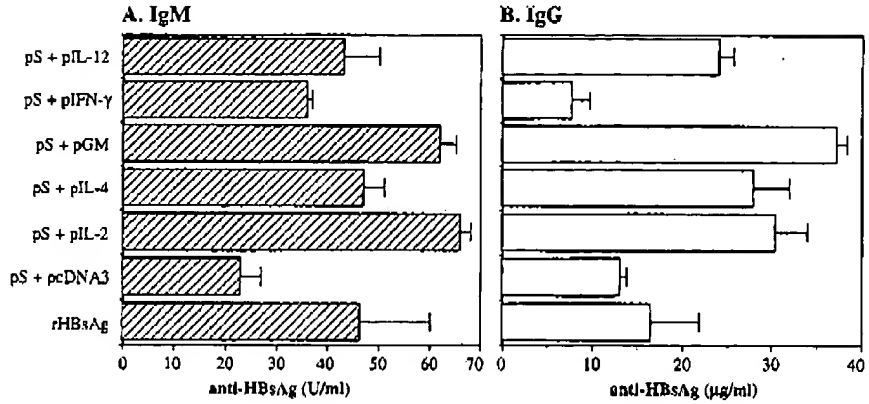

FIGURE 2. Effect of coinoculation with plasmids expressing various cytokines on HBsAg-specific Ab responses. Groups of five C57BL/6 mice were given i.m. injections of a mixture of 100 μg of pS plus 100 μg of various cytokine vectors or the control vector pcDNA3. For protein immunization controls, mice were injected s.c. with 4 μg of rHBsAg in CFA. Sera were collected at week 4 and 8 following vaccination for analysis of IgM and IgG anti-HBs Ab, respectively. The amounts of IgM and IgG anti-HBs Abs were measured from standard curves generated from the respective serially diluted control Abs and expressed as U/ml and μg/ml, respectively. The data are presented as mean ± SD for five animals per time point from one representative experiment of three performed.

IL-12 or IFN-γ with the HBV DNA vaccine resulted in a significant increase in Th1 cytokine production (Fig. 3, *A* and *B*); IFN-γ levels were increased six- to sevenfold, and IL-2 levels were increased about fivefold. This was accompanied with a complete shutoff of IL-4 production (Fig. 3*C*). In contrast, cells from mice coimmunized with pS and pIL-4 produced much more IL-4 and much less IL-2 and IFN-γ as compared with cells from the control mice, indicating a bias toward the Th2 immunity. Coinjection of plasmids pIL-2 or pGM enhanced development of T cells that produce IL-2 and IFN-γ in response to challenge with a specific Ag, while cells that produce IL-4 were not significantly affected. These results indicate that the specific Th cell populations induced by a DNA vaccine can be altered by local expression of cytokines. Coexpression of IL-12 or IFN-γ promotes Th1 and down-regulates Th2 development, coexpression of IL-4 promotes Th2 and down-regulates Th1 development, and coexpression of IL-2 or GM-CSF largely enhance Th1 development and to a lesser extent Th2 development.

IgG isotypes to HBV DNA vaccination can be modulated by coinjection of various cytokine genes

The patterns of Ab isotypes produced in response to immunization are reliable indicators of the types of cytokines produced in vivo. IgG2a is produced as a consequence of Th1 cell activation and IFN-γ secretion, whereas IL-4 enhances the production of IgG1 and suppresses IgG2a (46, 47). We therefore measured anti-HBs IgG isotypes in the sera of mice treated with pS and the plasmid encoding various cytokines at week 8 after immunization, the time point at which peak Ab titers were achieved in vaccinated animals. As shown in Figure 4, coexpression of IL-12 or IFN-γ resulted in a dramatic increase of anti-HBs IgG2a Abs, whereas titers of HBsAg-specific IgG1 Ab were decreased, indicating enhancement of Th1 and suppression of Th2 cell function in immunized animals. In contrast, mice treated with plasmids pS and pIL-4 showed an inhibition of anti-HBs IgG2a Ab, while the production of IgG1 Ab was increased, indicating suppression of Th1 and promotion of Th2 cell function in vivo. Animals coinjected with plasmids encoding IL-2 or GM-CSF showed a significant, five- to sevenfold, increase of anti-HBs IgG2a Ab, and a much lower increase of IgG1 Ab, indicating enhancement of Th1 cell function but no significant

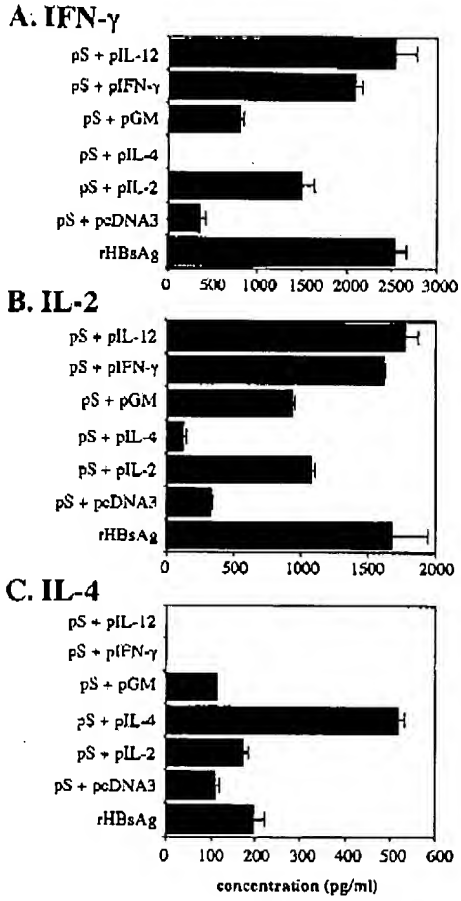

FIGURE 3. Effect of codelivery of various cytokine vectors on HBsAg-specific Th1/Th2 development in immunized animals. Animals were treated as described in the legend to Table I. At 2 wk after immunization, pooled splenocytes from each group were stimulated with 30 μg/ml of HBsAg. Culture supernatants obtained 3 days after stimulation were collected for quantitation of IFN-γ, IL-2, and IL-4 by ELISA.

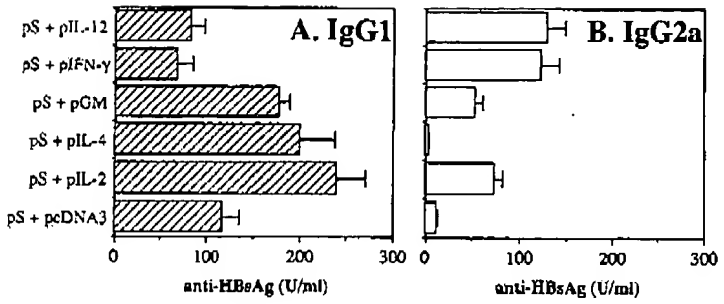

FIGURE 4. Effect of codelivery of various cytokine vectors on IgG isotypes of anti-HBs Ab. Animals were treated as described in the legend to Figure 2. Sera collected 8 wk after immunization were used to assay for the presence of IgG1 and IgG2a anti-HBs Abs. Concentrations of IgG1 and IgG2a anti-HBs Abs were measured from a standard curve generated from a serially diluted control Ab and expressed as U/ml. Data represent the mean titers ± SD of five animals per time point from one representative experiment of three performed.

change in Th2 cells. We also analyzed serum samples from mice given injections of pS and the cytokine vectors at different thighs and found that the levels of IgG1 to IgG2a isotypes were not altered (data not shown).

CTL activities induced by HBV DNA vaccination are affected by codelivery of cytokine genes

It is well known that cellular immunity is highly dependent upon the types of cytokines produced by Th cells. Thus, we tested whether the CTL activity induced by HBV DNA vaccine was influenced by coexpression of cytokines. Splenocytes from mice vaccinated with plasmids pS with or without a cytokine vector and restimulated in vitro were analyzed for their ability to lyse syngeneic cells expressing a transfected HBsAg gene (P815/S). Coinjection of pIL-2, pGM, pIFN-γ, or pIL-12 augmented HBsAg-specific CTL activity, in which enhancement by pIFN-γ or pIL-12 was consistently greater than that mediated by pIL-2 or pGM (Fig. 5*A*). In contrast, coinjection of pIL-4 substantially decreased the CTL activity as compared with that induced by pS without cytokine coexpression (Fig. 5*A*). The CTL activity was HBsAg specific, since mice immunized with pcDNA3 alone did not induce any detectable HBsAg-specific lysis and no CTL activity was observed in any of the groups of mice when parental P815 cells were used as target cells (data not shown). The effector cells that lysed HBsAg-expressing transfectants in vitro were eliminated by depletion of $CD8^+$ lymphocytes but not by depletion of $CD4^+$ lymphocytes (Fig. 5*A*). The different cytokine effect on the HBV DNA vaccine-induced CTL activity was further confirmed by assays using another HBsAg-transfected target cells, CT26/S, which produced more HBsAg than P815/S and was found to be more sensitive to the CTL assay. As shown in Figure 5*B*, HBsAg-specific cytolysis was enhanced by coinjection of the IL-12 gene but suppressed by the IL-4 gene (Fig. 5*B*). This cytolytic activity was blocked by an anti-CD8 mAb but not by an anti-CD4 mAb (Fig. 5*B*). Taken together, these results indicate that the CTL activity primed by a HBV DNA vaccine in vivo is mediated by cells expressing $CD4^-CD8^+$ surface phenotype and the CTL activity can be enhanced or suppressed depending on the cytokine gene coexpressed.

In vivo tumor immunity induced by HBV DNA vaccination is enhanced by coinjection IL-12 gene but suppressed by coinjection of IL-4 gene

To determine the effect of phenotype changes of immune responses on protective efficacy in vivo, the ability of the pS DNA vaccine in combination with IL-12 or IL-4 gene to immunoprotect against transplantation of syngeneic HBsAg-transfected tumor cells was examined. Groups of three to five mice were vaccinated once with 100 μg of pS plus 100 μg of pcDNA3 or vectors encoding IL-4 or IL-12. Mice that received pcDNA3 alone served as a negative control group. Three weeks after vaccination, all mice were inoculated with 1×10^5 HBsAg-expressing CT26/S or the parental CT26 tumor cells. All mice that received pcDNA3 and hence were not immune to HBsAg developed rapidly growing CT26 or CT26/S tumors within 2 wk after transplantation (Fig. 6,

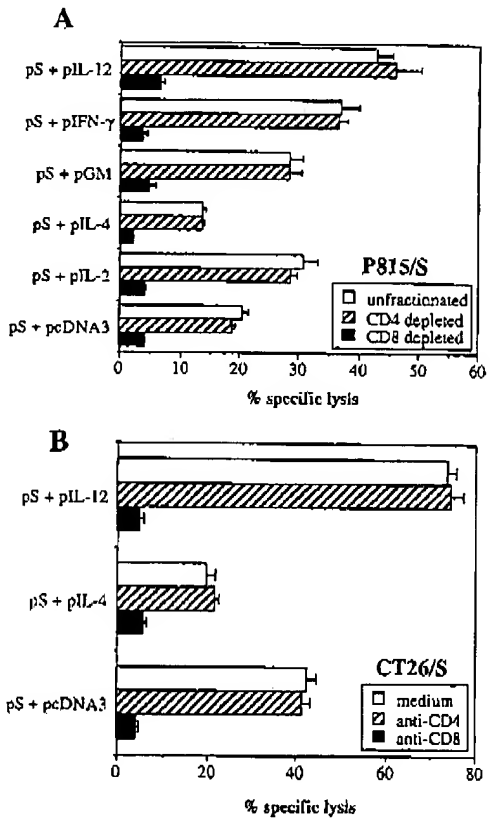

FIGURE 5. Effect of codelivery of various cytokine vectors on HBsAg-specific CTL activities. Groups of BALB/c mice were treated with plasmid pS plus a cytokine vector or a control vector as described in the legend to Table I. Three weeks after immunization, splenocytes were collected, cocultured for 4 days with irradiated P815/S transfectants, and used as effectors in the CTL assay. *A*, The specific cytolytic reactivity of unfractionated, $CD4^-$-depleted, or $CD8^-$-depleted effectors was tested on P815/S cells. *B*, The cytolytic reactivity of unfractionated effectors was tested on CT26/S cells in the presence of 10 μg/ml of anti-CD4 or anti-CD8 mAbs. The E:T ratio is 100:1 in all of these assays. Values of specific lysis are from one representative experiment of three performed and are presented as mean specific lysis of triplicate cultures ± SD.

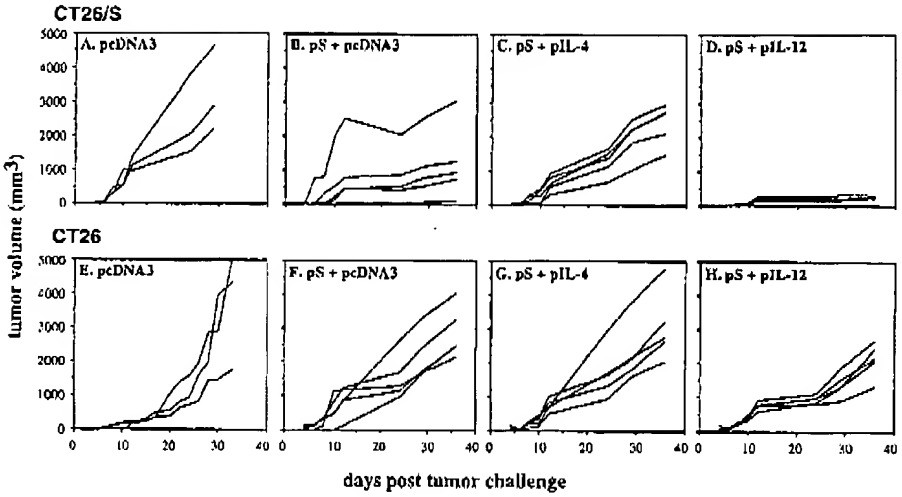

FIGURE 6. Effect of codelivery of various cytokine vectors on growth of CT26/S and CT26 cells. BALB/c mice were immunized with: 200 μg of control plasmid pcDNA3 (*A, E*); 100 μg of pS + 100 μg of pcDNA3 (*B, F*); 100 μg of pS + 100 μg of pIL-4 (*C, G*); or 100 μg of pS + 100 μg of pIL-12 (*D, H*). Three weeks after immunization, 1×10^5 CT26/S cells (*A–D*) or 1×10^5 CT26 cells (*E–H*) were transplanted s.c. into all mice. Growth of the tumor was measured every second or third day. The tumor volume from each animal at various time intervals after transplantation is plotted.

A and *E*). Four of five mice immunized with pS + pcDNA3 and challenged with CT26/S showed an inhibition of tumor growth (Fig. 6*B*). The protective efficacy was dramatically increased when the IL-12 gene was coinjected with plasmid pS; tumor growth was significantly suppressed, and two of five mice remained tumor free up to 60 days following tumor challenge (Fig. 6*D*). The tumor immunity is HBsAg specific, since mice immunized with pS + pcDNA3 (Fig. 6*F*) or pS + pIL-12 (Fig. 6*H*) and challenged with the parental CT26 cells showed little protection. In contrast, vaccination with pS + pIL-4 offered little if any protection against CT26/S (Fig. 6*C*) or CT26 (Fig. 6*G*) challenge. These results demonstrate that the difference in phenotype of immune responses stimulated by DNA vaccination under influence of various cytokines has a significant effect on protective efficacy against tumor challenge.

Discussion

Recombinant genes in the form of a plasmid expression vector can be simply injected and expressed in animal muscle (48, 49). Plasmids have been found to persist episomally in muscle cells, and gene expression in the skeletal muscle can be detected for up to 19 mo after injection (50). The injected DNA enter muscle cells through structures called T tubules and caveolae (51), and its nuclear transport likely occurs through the nuclear pore by a process common to large karyophilic proteins and RNA (52). Based on this technique, DNA vaccines with DNA instead of proteins in the vaccine formulation have been developed. Plasmid vectors containing appropriate target genes have been shown to induce humoral and cellular immune responses and protective efficacy in animal models of a variety of infectious diseases (4–15), cancers (16–21) as well as allergic (22–24) and autoimmune diseases (25). Compared with conventional protein-based vaccines, persistent expression of the encoded Ag by DNA vaccines would be expected to produce long-lived immunity. Indeed, mice that received a single injection of plasmid encoding HBsAg (53), influenza virus nucleoprotein (54), or hemagglutinin (55) have developed high levels of specific Ab that are persisted for at least 1 yr without significant reduction in titers. No evidence of insertional mutagenesis (56) or autoimmune responses, even in lupus-prone mice (57), can be raised after plasmid inoculation. However, neonatal immunization with DNA vaccines has been reported to induce tolerance rather than immunity (58).

Although the genetic vaccination approach has been applied to the development of DNA vaccines against many different pathogens, the efficacy of different DNA vaccines has varied widely. Some DNA vaccines are incapable of inducing specific immune responses even after several inoculations of large amounts of plasmid DNA (7, 43, 44). Thus, improvement of vaccine efficacy has become a critical issue for the acceptance of DNA vaccines as a standard vaccination technology. One explanation for the suboptimal immune responses induced by some DNA vaccines may be related to the cellular location of the encoded Ag. Indeed, it was found that the effectiveness of genetic immunization with a minigene coding for single epitopes was significantly enhanced if the epitope sequence was fused in frame with the adenovirus E3 leader sequence to target the epitope to the endoplasmic reticulum (59). Targeting of viral Ag for rapid cytoplasmic degradation was also reported to enhance de novo CTL responses in vivo (60). In contrast, it was reported that plasmids encoding a cytoplasmic/membrane or a secreted form of Ag have little effect on the ability of the plasmids to elicit humoral and cellular immune responses (33, 61, 62). Other approaches to enhance the efficacy of DNA vaccines include fusion of the encoded Ag with a stronger immunogen as a carrier (11), coexpression with B7-1/B7-2 accessory molecules (63, 64), or immunization with plasmid DNA-transfected dendritic cells (65).

Our laboratory has sought to promote and modulate immune responses to a DNA vaccine through codelivery of various cytokine genes. The rationale is based on our previous findings that a weak tumor Ag can be converted into a strong immunogen when it is conjugated to GM-CSF (39), IL-2, or IL-4 (38). It has also been reported that cytokines such as IL-1β, IL-2, or IFN-γ were able to enhance specific immune responses when administered along with the Ag for prolonged periods (66, 67). More direct evidence that cytokines can influence the efficacy of DNA vaccination was shown by Irvine et al. (68). These authors demonstrated

that, in a mouse tumor model, when recombinant IL-2, IL-6, IL-7, or IL-12 was added following administration of DNA encoding a tumor-associated Ag, the number of metastases was significantly reduced compared with that in mice treated with DNA only. To avoid the potential side effects associated with systemic administration of recombinant cytokines, we reason that direct injection of plasmids containing an Ag and a cytokine gene may be able to achieve a sustained but low level of cytokines, which are delivered to tissues in which immune interactions take place. Using this approach, we have shown previously that plasmids coexpressing IL-2 and HBsAg in the fusion or nonfusion context resulted in at least a 100-fold increase in the ability of the plasmids to induce Ab and T cell proliferative responses to HBsAg (33). In the present study, we attempt to accomplish local concentrations of cytokines by another strategy, that is, coinjection of cytokine genes with the HBV DNA vaccine. It was found that coinjection of the GM-CSF, IL-2, IL-12, or IFN-γ gene enhanced T cell proliferation by two- to threefold, while coadministration of the IL-4 gene achieved only a slight increase in this response (Table I and Fig. 1). Ab responses to the DNA vaccine were also affected by coexpression of cytokines. While GM-CSF, IL-2, IL-4, and IL-12 increased both IgM and IgG anti-HBs titers, IFN-γ was unique in that it increased IgM but suppressed IgG Ab (Fig. 2). We found that the adjuvant effect of the cytokine-expressing vectors was dependent upon coinjection with the plasmid encoding HBsAg; inoculation of the two plasmids separately had no effect on the magnitude of specific immune responses, indicating that colocalization of cytokines and Ags at the site of immune interaction was important for the observed cytokine adjuvant effect. It is noteworthy that the cytokine adjuvant effect is most significant within the first 2 to 5 mo following DNA vaccination and becomes negligible thereafter (data not shown), suggesting that the transfected muscle cells may be eliminated by that time. The enhancing effect of cytokine genes on immune responses to DNA vaccines has also been reported in other studies. In a rabies virus model, coinjection of plasmids encoding GM-CSF but not IFN-γ enhanced immune responses to the DNA vaccine (69). Similarly, plasmids encoding an idiotypic Ag of B cell lymphoma with its carboxyl-terminal end conjugated to GM-CSF or a nine-amino acid peptide derived from IL-1β were shown to induce enhanced Ab titers and to provide protective immunity against a subsequent lethal tumor challenge (19, 20). It has been reported recently that plasmid vectors containing an unmethylated CpG dinucleotide motif can elicit much stronger humoral and cellular responses to the encoded Ag than vectors that do not contain this sequence (42). The adjuvant effect of the cytokine vectors could be attributed to the presence of the CpG motif in the coding sequences of cytokines instead of functioning through their biologic effects. Our studies provide evidence that rules out this possibility, since coinjection of the control plasmid pcDNA3, which was used to construct the cytokine vectors, did not increase immune responses to the HBV DNA vaccine. Furthermore, none of the three potential immune-enhancing CpG motifs, i.e., GACGTC, AGCGCT, and AACGTT, was present in the coding sequence of murine IL-2, IL-4, GM-CSF, IFN-γ, or the IL-12 p40 genes. Among those tested, IL-12 p35 is the only gene containing one such motif; however, it is unlikely that this CpG motif by itself could make the immune phenotype changes observed in the IL-12 gene-injected mice.

Development of the appropriate $CD4^+$ Th cell subset during an immune response is critical for eradication of infectious organisms. A functional consequence of Th1 activation is the promotion of cell-mediated immune responses characterized by expression of C-fixing and opsonizing Abs, such as IgG2a in the mouse, as well as macrophage and CTL activation (28, 29). In contrast, Th2 cells promote development of selected humoral immune responses, including expression of IgG1 and IgE (28, 29). The mechanisms directing the development of naive CD4 cells into Th1 or Th2 effectors include the presence of certain cytokines at the site of primary Ag stimulation of naive cells, the effective concentration of Ag presented to T cells, and the nature of APC (70). Among these factors, the presence of local cytokines plays the most critical role in shaping the nature of immune responses. IL-4 is essential for priming Th2 cells (30), and IL-12 and IFN-γ strongly support the development of Th1 cells (31, 32). Most of the above conclusions were obtained from in vitro studies of naive Th cells stimulated with mitogen or Ag in the presence of various cytokines or from in vivo studies in which the exogenous cytokines were applied systematically. Codelivery of cytokine genes with the DNA vaccines can provide a sustained but low level of cytokines to tissues of immune interactions and may thus represent a more appropriate model for gaining insight into the differentiation of naive Th cells during an immune response. Our studies confirm the importance of IL-12 and IFN-γ for the development of Th1 cells and IL-4 for Th2 cells. Immunization of the HBV DNA vaccine with plasmids encoding IL-12 or IFN-γ produced T cells that make significant amounts of IL-2 and IFN-γ but no IL-4 in response to HBsAg (Fig. 3), indicating a shift toward Th1 immunity. This observation is corroborated by the in vivo increase of HBsAg-specific IgG2a Ab (Fig. 4*B*) and enhancement of CTL activity (Fig. 5, *A* and *B*). In contrast, coexpression of IL-4 induced a shift toward the production of Th2 cytokine (Fig. 3*C*), which is further confirmed by the in vivo increase of specific IgG1 production (Fig. 4A) and decrease of IgG2a (Fig. 4*B*) and CTL activity (Fig. 5, *A* and *B*). Coexpression of IL-2 or GM-CSF with the HBV DNA vaccine favored the generation of Th1 cells (Fig. 3, *A* and *B*); however, the Th2 cell response was less affected (Fig. 3*C*). This effect was accompanied by an enhanced production of both IgG1 (Fig. 4*A*) and IgG2a (Fig. 4*B*) isotypes as well as by CTL activity (Fig. 5*A*). We found that there was a strong correlation of CTL activity in immunized animals with protective efficacy against syngeneic HBsAg-transfected tumor cells. Mice that received a mixture of the HBV DNA vaccine and the control vector showed an inhibition of tumor growth (Fig. 6*B*). The protective efficacy was dramatically increased when the DNA vaccine was coinjected with the IL-12 gene, which helps elicit the strongest CTL activity among various cytokine genes tested. In contrast, vaccination with the DNA vaccine in the presence of the IL-4 gene, which favored Th2 response and suppressed CTL activity, abolished the protective immunity (Fig. 6*C*). These results indicate that, by codelivering DNAs encoding Ag and specific cytokines, it is possible to regulate the quantitative and qualitative nature of an immune response to make it more effective in dealing with pathogens. We also found that the in vitro CTL activity of the immunized splenocytes is specific for HBsAg and can be removed by depletion of $CD8^+$ cells (Fig. 5*A*) or by addition of an anti-CD8 mAb (Fig. 5*B*) in the culture medium, but not by depletion of $CD4^+$ cells or by the CD4-specific mAb, indicating that the CTL activity is mediated by $CD8^+$ lymphocytes. It was demonstrated that the CTL generated by HBsAg DNA immunization is restricted by class I MHC and the L^d-restricted S28–39 epitope is the only CTL epitope of HBsAg in $H\text{-}2^d$ BALB/c mice (71). Similar findings on the regulation of Th1 vs Th2 populations by the IL-2, IL-4, or GM-CSF gene in the immune response to a hepatitis C virus DNA vaccine has also been reported previously (72). In another study, Kim et al. have shown that codelivery of the IL-12 gene along with DNA vaccine formulations for HIV-1 Ag resulted in the reduction of specific Ab response, whereas the T cell-proliferative reaction and

CTL activity were increased (73). It is noteworthy that coexpression of the IL-12 gene with the HIV-1 DNA vaccine caused splenomegaly and increased the number of white blood cells in the immunized spleen, a phenomenon also reported by in vivo administration of rIL-12 (74–76). In our study, we found that codelivery of the IL-12 gene, as well as the other cytokine genes tested, did not lead to enlargement of the spleen (data not shown) or changes of frequency of $CD4^+$ and $CD8^+$ cells in the spleen. What caused the discrepancy between these two studies is not clear, but it may be due to the amount of IL-12 produced by the different IL-12 vectors.

HBV is one of several viruses that can persist after primary infection in humans, subsequently causing chronic necroinflammatory liver disease and hepatocellular carcinoma. The currently used therapeutic approaches to eliminating the virus and terminating chronic infections have not been very successful. It is widely acknowledged that, in humans, T cells play a critical role in clearing HBV infections and in inducing liver lesions associated with persistent HBV infections (77). Therefore, the idea of vaccinating HBV chronic carriers to control viral infections is challenged mainly due to a concern of possible massive liver damage mediated by immune cells. However, a recent report has shown that adoptively transferred HBsAg-specific CTL can abolish HBV gene expression and replication in HBV transgenic mice by secreting IFN-γ and TNF-α (78). Most importantly, these cytokines perform their antiviral activity mainly by a noncytopathic process. These results raise the possibility that chronic HBV infection may be controlled by induction of appropriate T cell subsets, which can produce the therapeutic cytokines. Indeed, using a similar transgenic model, it was reported that T cell-mediated immunity induced by a HBV DNA vaccine resulted in the complete clearance of circulating HBsAg and in the long-term control of transgene expression in hepatocytes (79). Again, no detectable cytopathic effect was found to accompany this therapeutic response. HBV DNA vaccine has also been shown to induce high titers of anti-HBs Ab in chimpanzees (80), indicating its potential application in human trials. According to the studies and data presented here, we believe that the use of DNA vaccines in combination with an appropriate cytokine gene to regulate the phenotype of immune responses may provide an effective treatment for chronic HBV infections.

Acknowledgments

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